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Smc5-Smc6-Dependent Removal of Cohesin from Mitotic Chromosomes[▽]

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The function of the essential cohesin-related Smc5-Smc6 complex has remained elusive, though hypomorphic mutants have defects late in recombination, in checkpoint maintenance, and in chromosome segregation. Recombination and checkpoints are not essential for viability, and Smc5-Smc6-null mutants die in lethal mitoses. This suggests that the chromosome segregation defects may be the source of lethality in irradiated Smc5-Smc6 hypomorphs. We show that in *smc6* mutants, following DNA damage in interphase, chromosome arm segregation fails due to an aberrant persistence of cohesin, which is normally removed by the Separase-independent pathway. This postanaphase persistence of cohesin is not dependent on DNA damage, since the synthetic lethality of *smc6* hypomorphs with a topoisomerase II mutant, defective in mitotic chromosome structure, is also due to the retention of cohesin on undamaged chromosome arms. In both cases, Separase overexpression bypasses the defect and restores cell viability, showing that defective cohesin removal is a major determinant of the mitotic lethality of Smc5-Smc6 mutants.

Three essential SMC (structural maintenance of chromosomes) complexes control chromosome dynamics: condensin, cohesin, and the Smc5-Smc6 complex (37). They are composed of SMC heterodimers: Smc2 and -4 in condensin, Smc1 and -3 in cohesin, and Smc5 and -6 in Smc5-Smc6. These are large ATPases with globular N and C termini, which are separated by long coiled-coil domains. The termini interact through an ABC-like coordination of ATP through Walker A and B motifs, with the coiled-coils bending at a flexible "hinge" that acts as the SMC dimerization domain. Each complex contains a number of unique non-Smc subunits, which are likely to contribute to its unique function. Among these is a kleisin subunit, which interacts with both the SMC subunits, closing a potential ring-shaped structure (55, 61).

Condensin is localized to chromosomes primarily during mitosis and is essential for mitotic chromosome condensation. Conversely, cohesin is localized primarily to interphase chromosomes and has been postulated to form a ring-shaped structure around sister chromatids to ensure their cohesion, which is important for DNA repair by homologous recombination (HR). As its name suggests, the function of the Smc5-Smc6 complex is relatively poorly understood.

Scc2/4 loads cohesin onto chromosomes in G_1, and sister chromatid cohesion is established during replication via the action of the acetyltransferase Eco1. Cohesin must be removed before chromosome segregation, where cleavage of the kleisin subunit Scc1 by the protease Separase is critical (51). In *Saccharomyces cerevisiae*, Separase-mediated Scc1 cleavage is essential for the removal of cohesin from all loci. In mammals, most cohesin is removed from chromosome arms early in mitosis in a Separase-independent process regulated by cohesin phosphorylation (28, 76). At anaphase, Separase-dependent removal of cohesin at the kinetochores ensures sister chromatid separation. In *Schizosaccharomyces pombe*, cohesin is thought to be regulated in a manner similar to that in mammals; only a small fraction of the Scc1 homolog Rad21 is cleaved by Separase (70), suggesting that most cohesin is removed by a Separase-independent mechanism.

Cohesin-mediated sister chromatid cohesion is required for HR (64). Cohesin is recruited to double-stranded DNA breaks (DSBs) (66) and enforces cohesion genome wide after DNA damage in *S. cerevisiae* (65, 74). The acetyltransferase activity of Eco1 is essential for genomewide damage-induced cohesion, acting via the acetylation of Smc3 (6, 73, 81). In human cells, small interfering RNA (siRNA) studies have suggested a requirement for Smc5-Smc6 to recruit cohesin to DSBs (57), but this is not the case in *S. cerevisiae* (65), so the functional relationship between these related complexes also remains to be determined.

In *S. cerevisiae*, Smc5-Smc6 is loaded onto chromatin by the cohesin loader Scc2/4 at loci that overlap with cohesin, including at DSBs (36). Smc5-Smc6-null mutants of *S. pombe* die in aberrant mitoses (27, 75), though the cause of this is unknown. Genetic analyses of Smc5-Smc6 in these yeasts have focused on its role in DNA repair by utilizing viable hypomorphic mutants that are highly sensitive to DNA damage. Studies with two hypomorphic *smc6* mutants, bearing the *smc6-X* and *smc6-74* mutations, have shown that Smc5-Smc6 is required for a late stage of HR subsequent to the recruitment of the Rad51/Rad52 recombination proteins and the formation of recombination intermediates (2). *smc6-74* is a mutation (A151T) in the arginine finger motif of the N-terminal globular domain, while *smc6-X* is a mutation (R706C) in the hinge domain. Overexpression of Brc1, a multi-BRCT domain protein, suppresses the DNA damage sensitivities of several Smc5-Smc6 mutants but does not suppress *smc6-X* (45, 75). *smc6-74* mutants, but not *smc6-X* mutants, are also defective in an early response to replication fork stalling, involving the recruitment of Rad52 but not Rad51 (30).

As with cohesin, the HR defects in Smc5-Smc6 hypomorphic

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mutants are likely to result from a more general role in chromosome organization than acting as a recombinase. Smc5-Smc6 is required for HR following irradiation or recovery from hydroxyurea (HU)-induced replication arrest (2, 18, 27, 34, 35, 71, 75). However, in contrast to the sustained checkpoint arrest of irradiated HR mutants, *S. pombe* Smc5-Smc6 hypomorphs, such as that with the *smc6-74* mutation, enter highly aberrant mitoses following DNA damage. For DSBs induced by ionizing radiation, *smc6* mutants progress into mitosis with wild-type kinetics, but, as shown by pulsed-field gel electrophoresis (PFGE), the chromosomes are highly fragmented (75). In each case, the mitotic defects are blocked by an earlier (upstream) HR defect (2, 27, 43). The chromosome segregation and recombination defects are apparent on each of the three *S. pombe* chromosomes and are not limited to the ribosomal DNA present on both ends of chromosome III.

These aberrant mitoses of Smc5-Smc6 mutants following DNA damage either block segregation completely (the "cut" phenotype, where the division septum bisects the nucleus) or result in partially segregated chromosomes that are incompletely resolved along the division plane, with an elongated mitotic spindle. Since Smc5-Smc6 is required to maintain a damage induced checkpoint arrest, the aberrant mitoses of Smc5-Smc6 mutants could result from attempting to segregate incompletely repaired chromosomes. Alternatively, defects may reflect a role for Smc5-Smc6 in promoting chromosome segregation that is revealed in hypomorphic mutants following exogenous DNA damage but is evident in null mutants without DNA damage or with low-level endogenous lesions. Notably, while viable, the hypomorphic mutants show a high level of spontaneous aneuploidy, which is also consistent with defects in chromosome segregation (35, 75).

Another characteristic of *smc6* mutants in *S. pombe* is a strong synthetic lethality with a temperature-sensitive (ts) allele of topoisomerase II (Top2), *top2-191*, at a permissive temperature for *top2-191* of 30°C. This lethality is due to a failure of chromosome segregation that resembles mitoses in irradiated *smc6-74* cells (75). *top2-191* is a A802V mutation (63), and cells with this mutation show no defects in cell cycle progression at 30°C. At 36°C, *top2-191* cells enter mitosis with normal kinetics but fail to segregate chromosomes. The defects of *top2-191* cells at the restrictive temperature of 36°C manifest exclusively in mitosis without an interphase delay and include defective chromosome condensation. Therefore, the *top2-191* allele may not affect the postreplicative decatenation activity of Top2 in *S. pombe*. Rather, the *smc6–top2-191* interaction may be related to the structural role played by Top2 in mitotic chromosome architecture (12, 14, 79).

In vertebrate cells, defective decatenation caused by Top2 inhibition with drugs such as etoposide or doxorubicin block the rejoining of molecules cleaved by Top2. This leaves DSBs that elicit a G_2 DNA damage checkpoint response in many cell types (13, 16, 17, 38). Conversely, human cells in which Top2 has been deleted enter mitosis but show disordered chromosomes that fail to segregate (12). Thus, in *S. pombe*, *top2-191* has a terminal phenotype more closely related to that of human cells with Top2 deleted than to that of cells with chemically inhibited Top2 that are blocked midway in the decatenation reaction.

Here we have investigated the mitotic role of Smc5-Smc6 in *S. pombe*. We find that Smc5-Smc6 is required for the removal of cohesin from damaged chromosome arms prior to anaphase and from undamaged chromosomes when the mitotic function of Top2 is compromised. We show that a defect in cohesin removal is a major determinant of lethality in *smc6* mutants and highlight the importance of coordinating Smc5-Smc6 and cohesin function in the maintenance of genome integrity.

MATERIALS AND METHODS

General *S. pombe* methods. All strains used were derivatives of *972 h*$^-$ and *975 h*$^+$. Standard procedures were used for propagation and genetic manipulation (44). Fluorescence-activated cell sorter (FACS) analysis for DNA content was performed with a FACSCalibur flow cytometer (Becton Dickinson) on 70% ethanol-fixed cells. Chk1 activation was assayed by Western blotting using a hemagglutinin (HA)-tagged *chk1* allele as described previously (11). Survival assays with methyl methanesulfonate, HU, and UV-C irradiation were performed as described previously (62). Where indicated, latrunculin B (1 mM stock in dimethyl sulfoxide; Biomol International) was added at 10 μM to inhibit cytokinesis. In the case of temperature shifts from 25°C to 30°C, latrunculin B was added 1 h after the temperature shift. Cells treated with UV-C irradiation (100 J/m^2) or released from HU (11 mM for 5.5 h at 25°C or 4 h at 30°C) were immediately shifted into a medium with latrunculin B. HU was removed by filtration, followed by extensive washing and reinoculation into fresh medium.

Microscopy. Microtubules were stained with anti-α-tubulin (clone B-5-1-2; 1:100; Sigma) and Cy3-conjugated anti-mouse immunoglobulin G (1:100; Sigma). DNA was visualized with 1 mg/ml 4′,6-diamino-2-phenylindole (DAPI). Postmitotic (anaphase plus binucleate G_1) cells were scored as those with segregated nuclear masses that were either incompletely resolved or fully resolved into binucleated cells. Data were collected from three samples of at least 100 cells. Images were captured on a Spot RT/SE camera using Spot advanced software and were cropped with Adobe Photoshop. Microscopy was performed with a Nikon E800 microscope and a 100×/1.40 Plan-Apo objective lens.

PFGE. Cells were washed in ice-cold Stop Buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3) prior to processing for PFGE as described previously (3). Samples were run on 0.8% chromosome-grade agarose (Bio-Rad)–Tris-acetate-EDTA gels for 72 h, with a pulse time of 1,800 s, at 2 V/cm and an angle of 100°.

DNA damage markers. Western blotting for anti-HA (12CA5) was used to detect activated HA-tagged Chk1, which migrates as a higher-molecular-weight species (77). Phosphorylated H2A (γ-H2A) was detected with a rabbit anti-γ-H2A antibody (ab17353; 1/2,000; Abcam). Actin and Cdc2 were used as loading controls. Yellow fluorescent protein-tagged Rad22 (the Rad52 homolog) expressed from the *rad22* locus was crossed into the relevant backgrounds and was visualized directly in live cells.

Visualization of LacI-GFP-marked loci. For centromere 1, a strain in which a LacO array is integrated at *lys1*, close to centromere 1, and which expresses green fluorescent protein (GFP)-LacI from the *nmt1* promoter (69) was crossed into the relevant backgrounds. Cells were grown in minimal medium containing 0.5 μM thiamine; at 1 μM thiamine, GFP-LacI was not visible, whereas in the absence of thiamine, GFP is present throughout the nucleus. Occasional aggregation of GFP was seen in ~5% of cells, which were excluded from analysis. *top2-191* and *smc6-76 top2-191* cells were induced for 22 h at 25°C and then shifted to 30°C for 4 h (the last 3 h with 10 μM latrunculin B). Wild-type and *smc6-74* cells after HU recovery were induced at 30°C for a total of 19 h. HU at 11 mM was added after 11 h, and cells were incubated for an additional 4 h. Cells were then washed and reinoculated into a medium containing latrunculin B for an additional 4 h. For the arm loci, strains contained the same LacO array integrated at *his2*, *ade8*, *ade6*, and *ade1*, with GFP-LacI constitutively expressed from the *his7* promoter (19, 25, 80). Exponentially growing cells were processed as described above for the shift to 30°C (*top2-191* and *top2-191 smc6-74* cells) or recovery from HU-induced arrest (wild-type and *smc6-74* cells). Data were collected from three samples of 50 cells. All cells were fixed in methanol before the number of GFP foci present in cells with single nuclei was scored.

Chromosome condensation. Cells were arrested in metaphase by overexpression of Mad2 from the *nmt1* promoter. Expression of Mad2 was induced at 25°C for 22 h, and cells were then shifted to 36°C for 4 h prior to fixation in 3.7% formaldehyde.

Construction of *top2-Y835F*. The codon encoding the catalytic tyrosine (TAT) of Top2 cloned into pREP81 (39) was mutated to phenylalanine (TTT) by the site-specific mutagenesis method of Kunkel et al. (32) with the oligonucleotide

TABLE 1. Oligonucleotides used for ChIP assays

Locus[a]	Oligonucleotide sequence	
	Forward	Reverse
Centromeres (*otr*)	GCGTCGGAAGGTTGAGAATA	CTGCACTAGCAATTGGATCG
1L (*pac2*)	CGCTCAACTGTCTTGCAAAT	CGATATTCGCAGACGAAACA
1R (*fun14*)	TTCCTCTTGGCAATTGGAGT	AATGCCAAGCGCTATTCAAC
2L (*act1*)	CCATTGAGCACGGTATTGTC	AGGAGCCTCAGTCAACAAGC
2R.1 (*zfs1*)	CTCTAGCACAGCACGTGAGC	ATCCGGATTGTGGTCTTCAG
2R.2 (*pyr1*)	CATGAGCAGTTCGGAAGACA	CAGGATCGTTCCTCCTTGTT
2R.3 (SPBP4H10.14)	TAGTCCGCGTTCTTCGTTCT	GCACATTGAAACCGGACTGT
3L (SPCC553.10)	CTTGCTGCTTCCGCTAAACT	GCTGAACGACCGTCTTGTTT
3R (*ade6*)	TTAAGCTGAGCTGCCAAGGT	GGCTGCCTCTACCATCATTC
his3 (5′ to *MAT***a**)	CGTAACAGTTGCTGGCGTTT	GAAACCGTATGCAGAACTGGAG
kanMX6 (3′ to *MAT***a**)	CAATCAGGTGCGACAATCTATC	ATCATTGGCAACGCTACCTT

[a] The closest gene is given in parentheses.

AGCTGTATTTAGAAACCTTGAAGCCGATGC. Complementation was assayed in the presence of thiamine.

Separase overexpression. pCut1 is the *cut1* open reading frame cloned into pREP81 (39). DNA damage sensitivity was assayed on a medium lacking thiamine. A protease-deficient Cut1 allele (C1730A) was made using the site-specific mutagenesis method of Kunkel et al. (32) with the oligonucleotide ATACAAA GCACCAGAGCTAGCACCCATCAGTATAGT.

ChIP. A GFP-tagged allele of Rad21 was crossed into the relevant backgrounds, and chromatin immunoprecipitation (ChIP) was performed on 50-ml samples as described previously (2), with the exclusion of dimethyl adipimidate from fixation, using rabbit polyclonal anti-GFP antibodies (A11122; Molecular Probes) and protein G Dynabeads (Invitrogen). FACS was used to ensure that samples were in G_2 prior to the inactivation of Mis4. ChIP primers are described in Table 1 and were designed with Primer 3 software (59). Data were generated by quantitative PCR (Opticon 3; MJ Research) and represent means ± standard errors (SE) ($n = 3$ to 5).

Induction of a unique DSB. A *MAT***a** site was inserted with a *kanMX6* cassette into the *his3* locus. A ts allele of *HO* was generated by random mutagenesis of *HO* cloned into pREP3 (39) by propagation in *Escherichia coli* XL1-Red (Stratagene), and cells were screened for this allele by temperature-dependent lethality in an *rhp51*Δ background. ts HO was expressed from the *nmt1* promoter at 36°C for 20 h, and cells were shifted to 25°C for 2 h to induce a DSB. ChIP for Rad21-GFP was then performed on both sides of the break.

RESULTS

Chromosome segregation defects in *smc6-74* cells. Most genes of the Smc5-Smc6 complex are essential. Mutants with null alleles or strong conditional loss-of-function mutants die in lethal mitoses that are exacerbated by DNA damage in the preceding interphase. Hypomorphic mutants, such as *smc6-74* mutants, also die in lethal mitoses following DNA damage in the previous interphase. *smc6-74* is synthetically lethal, due to mitotic failure, with the ts topoisomerase II mutation *top2-191* at the permissive temperature (30°C) for *top2-191* (75). This is also true of other Smc5-Smc6 complex mutants and is not specific for this *smc6* allele (Fig. 1A and B).

Because Smc5-Smc6 is required for HR and checkpoint maintenance, we previously proposed that such mitotic defects were due to mitotic progression with unrepaired lesions (27). However, given that Smc5-Smc6 genes are essential for viability and those for HR are not, we could not rule out the possibility that these mitotic defects reflect a role for Smc5-Smc6 in chromosome segregation.

To address this possibility, we undertook a detailed analysis of the synthetically lethal mitoses of *smc6-74 top2-191* cells seen at 30°C without exogenous DNA damage. These aberrant mitoses display the cut phenotype, in which the division septum lethally bisects the unsegregated or incompletely resolved chromosomes (Fig. 1C). We asked if chromosome segregation was merely delayed and whether the chromosomes would eventually segregate in *smc6-74 top2-191* cells if cytokinesis (and hence nuclear cutting) were inhibited. To this end, the actin poison latrunculin B was used at 10 μM. At this concentration, latrunculin B has been reported to delay mitosis when added to yeast extract-peptone-dextrose medium (23). However, we observed no delay of mitotic progression in our experiments, since ~80% of wild-type cells became binucleate in one cell cycle at 30°C (Fig. 1C), but cytokinesis was inhibited. This may reflect differences in the potency of latrunculin B preparations and/or the fact that all our experiments are performed in the defined minimal medium EMM2 (44).

Among the wild-type and single mutant cells treated with latrunculin B, ~80% become binucleate (Fig. 1C). By FACS analysis, we observed that many of these cells underwent another round of DNA replication to become 4C (that is, the cells now contained two G_2 nuclei, each with a 2C DNA content) (Fig. 1D). In *top2-191 smc6-74* cells, latrunculin B prevented the lethal "cutting" of unsegregated or incompletely resolved chromosomes by the septum. However, only ~25% of cells become binucleate (the majority showed no chromosome segregation and remained uninuclear), yet 50% of cells became 4C (Fig. 1D). That is, DNA rereplication was occurring without chromosome segregation, indicating that the chromosomes are likely intact, since persistent lesions should block entry into S phase (52, 53, 72).

We next tested if the synthetic lethality of *top2-191 smc6-74* mutants was due to a catalytic defect in Top2-191. Surprisingly, growth at 30°C was rescued by expression of a catalytically inactive (Y835F) *top2* mutant (Fig. 1E), suggesting that the lethality might be due to a defect in the structural role played by Top2 in mitotic chromosome architecture (12, 14, 79) rather than to a defect in chromosome decatenation. Top2 is also required for chromosome condensation, but the chromosome condensation defect of *top2-191* mutants at 36°C is not evident at 30°C, and the characteristic theta-shaped nuclei of condensation mutants (60) are not seen in *smc6-74 top2-191* cells (75). Moreover, metaphase-arrested *smc6-74 top2-191* chromosomes also condense at 30°C (Fig.

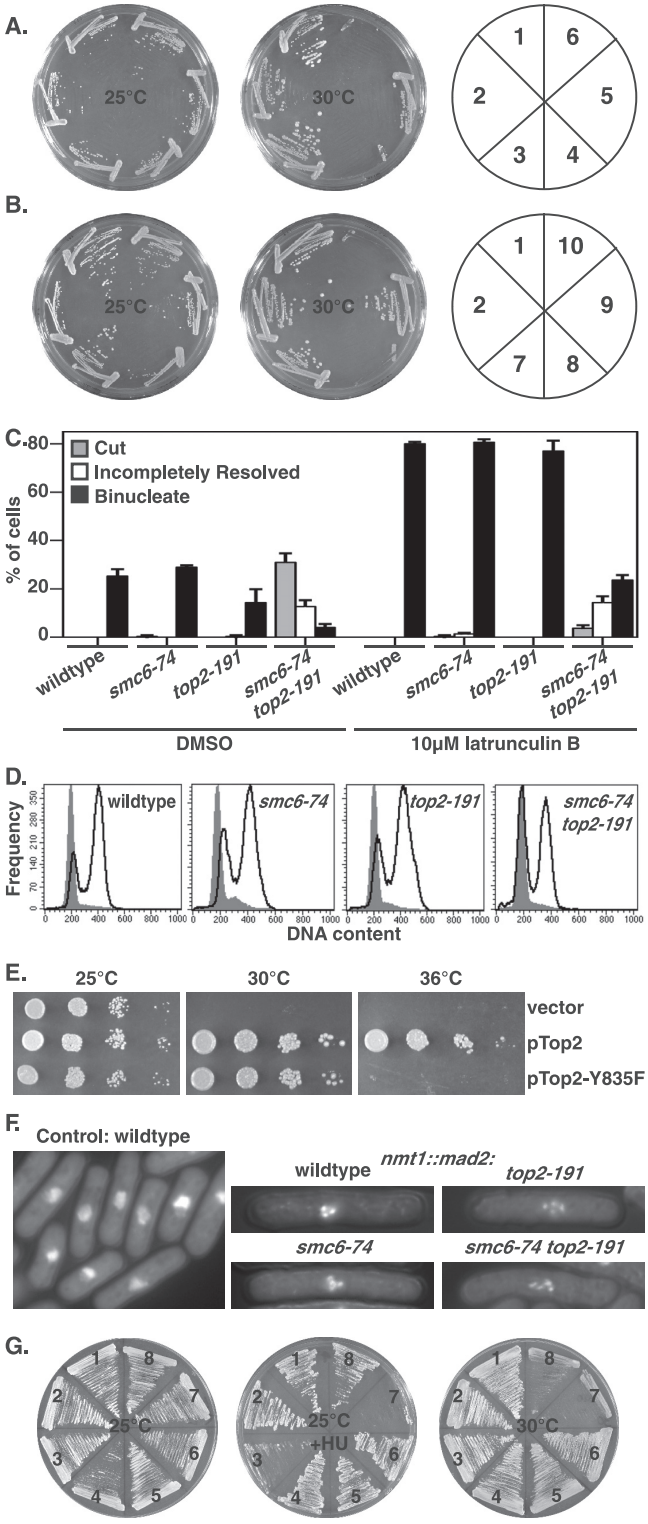

FIG. 1. Smc5-Smc6 and Top2 cooperate in chromosome arm segregation. (A and B) Synthetic lethality between Smc5-Smc6 mutants and *top2-191* mutants. Plates were incubated at 25°C (5 days) and 30°C (4 days). (A) Numbered sections contain strains with the following genotypes: 1, wild type; 2, *top2-191*; 3, *smc6-74*; 4, *smc6-74 top2-191*; 5, *smc6-X*; 6, *smc6-X top2-191*. (B) Numbered sections contain strains with the following genotypes: 1, wild type; 2, *top2-191*; 7, *nse2-SA*; 8, *nse2-SA top2-191*; 9, *rad60-1*; 10, *rad60-1 top2-191*. All double mutants are lethal at 30°C. (C) Strains were grown at 25°C and shifted to 30°C for 4 h with either 10 μM latrunculin B (to block cytokinesis) or dimethyl sulfoxide (DMSO) as a control and were scored for cells in aberrant mitoses (cut or incompletely resolved) or cells successfully completing mitosis (binucleate). The remaining percentages of cells were uninuclear. In the presence of latrunculin B, chromosome segregation was blocked in *smc6-74 top2-191* cells, the majority of which remained uninuclear. Data are means ± standard deviations for three samples of 100 cells. (D) DNA profiles of the cultures shown in panel A that were grown at 25°C (2C) (shaded histograms) or for 4 h at 30°C with 10 μM latrunculin B (2C and 4C) (open histograms). *smc6-74 top2-191* cultures became ~50% 4C despite the failed chromosome segregation (~25% binucleate, versus ~80% in controls). (E) *smc6-74 top2-191* cells were transformed with either pREP81 (vector), pREP81 containing wild-type *top2* (pTop2), or pREP81 containing catalytically inactive *top2-835F* (pTop2-Y835F). Tenfold serial dilutions were spotted onto plates and grown at the indicated temperatures for 4 days. Catalytically inactive Top2 rescued the synthetic lethality of *smc6-74 top2-191* cells at 30°C. (F) Analysis of chromosome condensation at metaphase. DAPI images of the indicated strains with vector controls show normal uncondensed nuclei (left) and metaphase-arrested cells (Mad2 overexpression) with condensed individual chromosomes in all strains (right). (G) The indicated strains expressing Brc1 from pREP41 (pBrc1) and vector controls were streaked onto medium with or without 4 mM HU for 5 days (25°C) or 4 days (30°C). Strains contain vector only (odd numbers) or pBrc1 (even numbers). Numbered sections contain strains with the following genotypes: 1 and 2, wild type; 3 and 4, *smc6-74*; 5 and 6, *top2-191*; 7 and 8, *smc6-74 top2-191*. pBrc1 rescues the HU sensitivity of *smc6-74* and *smc6-74 top2-191* cells at 25°C but fails to rescue the synthetic lethality of *smc6-74 top2-191* cells at 30°C.

1F), suggesting that defective condensation is not contributing to the phenotype.

Brc1 is a BRCT domain protein that, when overexpressed, suppresses the sensitivity of *smc6-74* cells to a range of DNA-damaging agents that inflict lesions in S phase and in G_2, even when these agents are used at concentrations ordinarily resulting in <1% survival of *smc6-74* cells (33, 62, 75). We therefore reasoned that if the source of the mitotic defects were unrepaired lesions, Brc1 overexpression should rescue the *top2-191 smc6-74* synthetic lethality. This, however, is not the case; there was no rescue of growth at 30°C, but Brc1 did rescue the HU sensitivity of *smc6-74* and *smc6-74 top2-191* cells at 25°C (Fig. 1G).

Absence of detectable DNA damage in *top2-191 smc6-74* cells. The cellular phenotypes described above suggest that there may not be significant levels of DNA damage in *top2-191 smc6-74* cells at 30°C, and thus the synthetic lethality may not be due to a defective response to DNA damage. To test this notion more rigorously, we employed several additional, more-sensitive assays for DNA damage.

rhp51 encodes the *S. pombe* homolog of Rad51, and because *rhp51*Δ cells cannot undergo HR, they are very sensitive to DNA damage. In response to even low levels of DNA damage, *rhp51*Δ cells activate the DNA damage checkpoint, and because repair does not occur, this checkpoint remains engaged and the cells die in a lethal cell cycle arrest. *rhp51*Δ *smc6-74* double mutants have the same sensitivity as *rhp51*Δ mutants; that is, these alleles are epistatic. However, while *smc6-74* cells fail to maintain a DNA damage-induced checkpoint arrest, the checkpoint remains engaged following DNA damage in *rhp51*Δ *smc6-74* cells (2, 35, 43, 75).

We repeated the experiments for which results are shown in

Fig. 1C and D, in which chromosome segregation and genome rereplication are assayed in cells that cannot undergo cytokinesis. Because the lack of *rhp51* is a very sensitive in vivo gauge of DNA damage, the presence of even low levels of DNA damage in *top2-191 smc6-74* cells at 30°C should result in prolonged cell cycle arrest. We found that, on the contrary, *top2-191 rhp51*Δ *smc6-74* triple mutants continued to enter mitosis at 30°C but then failed to segregate sister chromatids, similarly to *top2-191 smc6-74* double mutants (Fig. 2A). Like the *top2-191 smc6-74* double mutant (Fig. 1D), the triple mutant also underwent another round of DNA replication to become 4C (Fig. 2B). Hence, if lesions do exist in *top2-191 smc6-74* cells at 30°C, either they do not require *rhp51* and hence HR for their repair or they are below a threshold required to elicit a checkpoint response to block entry into mitosis or into the subsequent S phase.

DNA damage in asynchronously growing *S. pombe* cultures causes cells to elongate during a G_2 cell cycle arrest, which is accompanied by phosphorylation of the checkpoint kinase Chk1 (54) and the C terminus of histone H2A (49) (γ-H2A), analogous to the phosphorylation of H2A-X in higher organisms (20). However, at 30°C, *smc6-74 top2-191* cells did not delay in G_2 (75), nor was Chk1 or H2A phosphorylated above background levels (Fig. 2C). Rad22, the *S. pombe* Rad52 homolog, is rapidly recruited to sites of DNA damage, which it can continue to occupy following repair (29). We observed no elevation in the percentage of cells with Rad22 foci in *top2-191 smc6-74* mutants above those in the parental strains at 30°C. Each of these markers was strongly induced by UV-C irradiation at 100 J/m^2, which kills ~70% of *smc6-74* cells (75). Finally, the *top2-191 smc6-74* chromosomes were resolved by PFGE (Fig. 2E), indicating an absence of DSBs (lower-molecular-weight fragments [75]), unresolved catenates, and recombination intermediates (both of which fail to enter the gel [2, 42]) in these cells grown at 30°C, despite a strong block to sister chromatid separation. Hence, the block to segregation is likely to be proteinaceous rather than to be due to intermolecular DNA interactions of recombination intermediates, because the block is removed during the preparation of these samples for PFGE, which includes the removal of proteins with proteinase K (3).

We conclude that despite mitotic failure, *top2-191 smc6-74* cells do not contain significant levels of DNA damage at 30°C. This is in keeping with the fact that *top2-191* cells show no evidence of DNA damage even at 36°C (75). Therefore, unrepaired DNA damage is unlikely to be the source of the block to mitosis in *top2-191 smc6-74* cells, and by extension, it may not be the cause of mitotic failure in *smc6-74* cells following extrinsic DNA damage.

The chromosome segregation defect in *smc6-74* is a postanaphase failure of sister chromatid arm separation. Smc5-Smc6 is required for recombination following replication fork collapse in order to restart replication. Following recovery from an HU-induced S-phase arrest, some replication forks spontaneously collapse, leading to the formation of recombination foci (2). Smc5-Smc6 is required for the resolution of recombination intermediates subsequent to the recruitment of Rad51 and Rad52 and the formation of joint molecules (2). In addition, Smc5-Smc6 is required for an "early" response involving the recruitment of Rad52 to stable, stalled replication forks

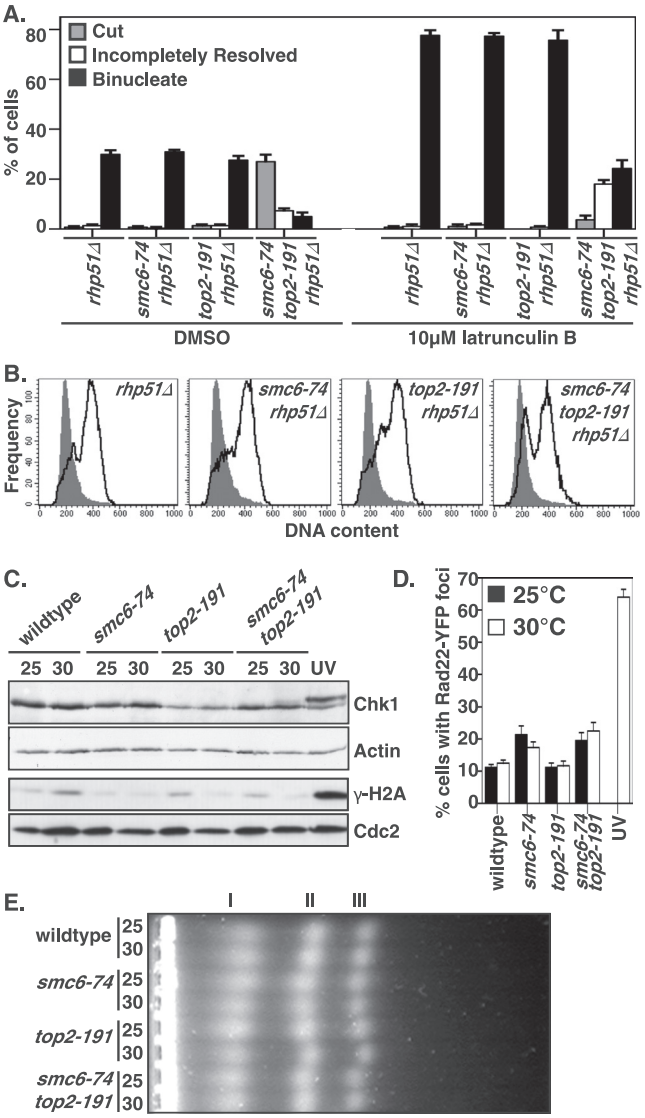

FIG. 2. The synthetic lethality of *top2-191 smc6-74* mutants is not associated with DNA damage. (A) The indicated strains in an *rhp51*Δ background were grown as for Fig. 1A and were processed for microscopy. The *rhp51*Δ mutation does not alter the kinetics of passage of *top2-191* cells through mitosis or the aberrant mitoses of *top2-191 smc6-74* cells. (B) DNA profiles of the cultures shown in panel A that were grown at 25°C (2C) (shaded histograms) or for 4 h at 30°C with 10 μM latrunculin B (2C and 4C) (open histograms). The *rhp51*Δ mutation does not prevent rereplication in *top2-191 smc6-74* cells, but these FACS profiles have more noise than those in Fig. 1D due to the nonuniform lengths of *rhp51*Δ cells. (C) Western blotting of cells grown at 25°C or shifted to 30°C for 4 h for Chk1 or γ-H2A. A UV-C-irradiated (100 J/m^2) sample was used as a control for phosphorylated (activated) Chk1 and γ-H2A. Actin and Cdc2 served as loading controls. (D) Yellow fluorescent protein (YFP)-Rad22 recombination foci were visualized in live cells grown at 25°C or shifted to 30°C for 4 h. A UV-C-irradiated (100 J/m^2) sample was used as a control for damage-induced foci, which remain following the completion of DNA repair (29). (E) PFGE of strains grown at 25°C or shifted to 30°C for 4 h. I, II, and III indicate the positions of normally migrating chromosomes.

(30), keeping these forks in a recombination-competent conformation.

Cytokinesis is dependent on the initiation of anaphase (40). The formation of septa during the aberrant mitoses in *smc6-74*

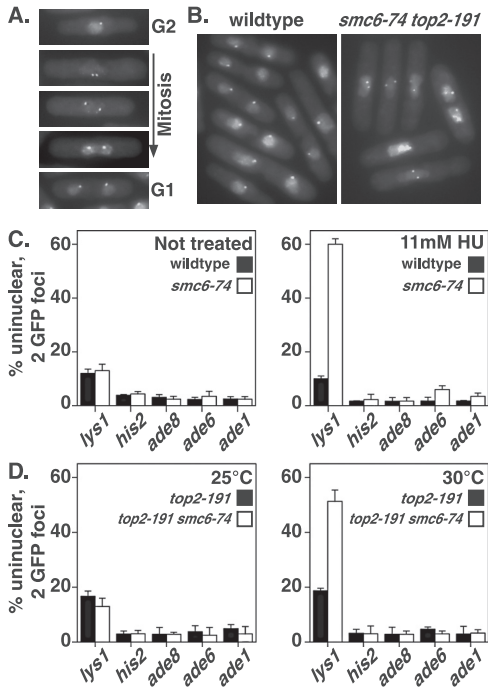

FIG. 3. Aberrant mitoses are due to a failure of chromosome arm segregation. (A) GFP-LacI foci in wild-type cells at the indicated phases of the cell cycle. (B) Examples of fields from wild-type and *smc6-74 top2-191* cells grown at 25°C and shifted to 30°C for 4 h (the last 3 h in the presence of 10 μM latrunculin B). A high percentage of wild-type cells are binucleate with single foci (normal mitotic exit to G_1), whereas *smc6-74 top2-191* cells have single nuclei with two foci, indicating kinetochore separation without chromosome segregation. (C and D) Only cells with single nuclei were scored for GFP foci. Cells with GFP aggregates (~5% of total) and GFP-negative cells were excluded from the analysis. (C) Quantification (means ± standard deviations from three samples of 50 to 100 cells) of GFP-LacI foci in uninuclear wild-type or *smc6-74* cells at LacO arrays integrated at the indicated loci. Cells were grown at 30°C (not treated) or released from a 4-h arrest in 11 mM HU. Samples were collected after a 4-h incubation in 10 μM latrunculin B. (D) *top2-191* and *top2-191 smc6-74* cells were grown at 25°C and either treated with 10 μM latrunculin B (left) or shifted to 30°C for 4 h in the presence of 10 μM latrunculin B (right). Uninuclear cells with two GFP foci were quantified as for panel C. In both cases, the defects in chromosome segregation occur despite kinetochore separation.

cells after DNA damage, and in *smc6-74 top2-191* cells at 30°C, indicates that the chromosome segregation defect occurs post-anaphase. This is corroborated by the elongation of mitotic spindles in cells with incompletely resolved chromosomes, though spindles are lost upon the lethal cutting of the nuclei (data not shown). We therefore assayed whether kinetochore separation was occurring by using GFP-LacI bound to LacO arrays integrated at *lys1*, close to centromere 1 (69). In this experiment, we also used 10 μM latrunculin B to block cytokinesis. Note that the majority of control cells become binucleate and that each nucleus has a single GFP focus, again showing that mitotic progression is not impaired in these experiments (Fig. 3A and B).

Wild-type and *smc6-74* cells were arrested in S phase with HU for 4 h and were then released into fresh medium containing latrunculin B. We determined the percentage of cells that contained a single nucleus but had two GFP foci resulting from kinetochore separation. Despite mitotic failure, kinetochore separation proceeded in HU-treated *smc6-74* cells (Fig. 3C). We carried out the same experiment with *top2-191* and *top2-191 smc6-74* cells grown at 25°C or shifted to 30°C for 4 h in the presence of latrunculin B. Again, kinetochore separation occurred despite the absence of chromosome segregation (Fig. 3D). This suggested that, under both conditions, there was a defect in chromosome arm segregation. We tested this with LacO arrays integrated at four independent arm loci, and indeed these loci failed to separate into two LacI-GFP foci (Fig. 3C and D). Therefore, Smc5-Smc6 is required for the separation of chromosome arms following DNA damage in the preceding interphase, and in the absence of detectable DNA damage (Fig. 2) when a mitotic function of Top2 independent of its catalytic activity (Fig. 1E) is compromised.

Separase overexpression suppresses the mitotic defects of the *smc6-74* mutant. We then screened for genes that, when overexpressed, restored mitotic progression after DNA damage to *smc6-74* mutants and to *smc6-74 top2-191* double mutants at 30°C. This experiment utilized genomic and cDNA libraries, as well as candidate genes involved in chromosome segregation, encoding the Polo (*plo1*)- and NIMA (*fin1*)-related kinases and the cohesin regulators Separase (*cut1*) and Securin (*cut2*). We found that ectopic expression of *cut1* from the weakest *nmt1* promoter (4) restored both chromosome segregation and colony formation to *smc6-74* cells in the presence of HU and also suppressed the synthetic lethality of *smc6-74 top2-191* mutants at 30°C (Fig. 4). Ectopic expression of Cut1 was itself lethal to *top2-191* cells at 30°C (Fig. 4B; also see below) and is lethal to wild-type cells if expressed from stronger promoters. The suppression of the mitotic defects of the *smc6-74* mutant was dependent on the protease activity of Cut1; the protease-dead C1730A mutant (47) did not suppress the mitotic defects under either condition and was not lethal to *top2-191* cells. The only other genes found to be high-copy-number suppressors in these screens were *smc6* itself and the previously characterized *smc6-74* suppressor *brc1* (75); each was isolated multiple times.

The most widely characterized function of Separase is the cleavage of the kleisin subunit (Rad21 in *S. pombe*) of cohesin complexes at anaphase (51, 61). However, Separase has also been implicated in other cell cycle events in *S. cerevisiae.* It has been shown to play a role in spindle elongation (5), though this is not defective in *smc6-74* cells (data not shown). Separase also has a nonproteolytic function in mitotic exit (68) in *S. cerevisiae*, but the suppression of the *smc6-74* defects is protease dependent. Finally, Separase has also been shown to cleave a kinetochore protein, Slk19, during mitosis. However, by iterative BLAST searches, this protein is not conserved in *S. pombe*, and a noncleavable Slk19 mutant does not block chromosome segregation (67).

Aberrant retention of cohesin on postanaphase chromosome arms following DNA damage in *smc6-74* cells. We next investigated if the mitotic defects in *smc6-74* cells following DNA damage are a result of cohesin dysregulation that blocks sister chromatid separation. This need not reflect a defect in Separase function per se in *smc6-74* cells, since Cut1 overexpression may bypass another defect in Separase-independent regulation of cohesin that restores sister chromatid separation.

We assayed cohesin localization by ChIP using Rad21-GFP

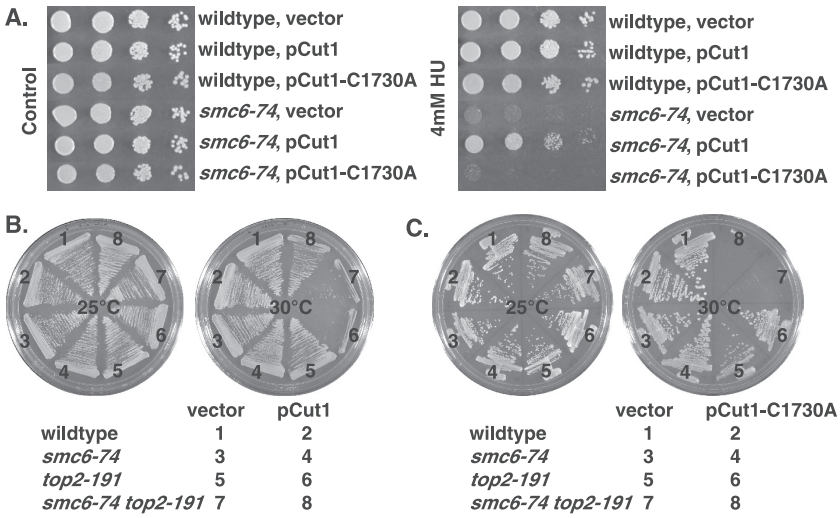

FIG. 4. Separase overexpression suppresses *smc6-74* defects. (A) Ectopic expression of Cut1, but not that of protease-dead Cut1-C1730A, suppresses the HU sensitivity of *smc6-74* cells. Tenfold serial dilutions were spotted onto plates and incubated at 30°C for 4 days. (B) Ectopic expression of Cut1 suppresses the synthetic lethality of the *top2-191 smc6-74* mutations at 30°C. Plates were incubated for 4 days. Note that Cut1 overexpression is lethal to *top2-191* cells at 30°C (section 6). (C) Ectopic expression of protease-dead Cut1-C1730A does not suppress the synthetic lethality of the *top2-191 smc6-74* mutations at 30°C. Plates were incubated for 4 days. Cut1-C1730A overexpression is not lethal to *top2-191* cells at 30°C (section 6).

and primers for the outer centromeric repeats (*otr*) and several loci on each chromosome arm. In synchronized cultures exiting mitosis (i.e., binucleate cells), loss of chromosomal cohesin was not observed, since cohesin is very rapidly reloaded following anaphase. However, if Mis4 (the Scc2 homolog, required to load cohesin in G_1) was inactivated postreplicatively using the ts *mis4-242* allele (22), chromosomal cohesin was lost after mitotic exit (Fig. 5), and this enabled us to assay the kinetics of cohesin loss as cells passed through anaphase.

Using a *mis4-242* background, cells were arrested in S phase with HU at 25°C, and upon removal of HU, cells synchronously completed S phase (Fig. 5B) and passed through mitosis (Fig. 5C). (In this experiment, HU acts as both a synchronizing and a DNA-damaging agent [2].) During the subsequent G_2 phase, half the culture was shifted to 36°C (to inactivate Mis4), and cohesin localization was assayed after exit from mitosis (Fig. 5D).

In an otherwise wild-type background (*mis4-242 rad21*::*GFP* cells), cohesin was lost from all loci once the cells passed through mitosis (Fig. 5D, top). However, in an *smc6-74* background, cohesin was retained at arm loci in HU-treated samples to ~80% of untreated levels (Fig. 5D, bottom), even though these cells passed through anaphase with the same kinetics as wild-type cells (2) (Fig. 5C). Note that three-quarters (33/44%) of the mitotic population at the final time point (270 min) in HU-treated *smc6-74* cells were either cut or incompletely resolved (11% were binucleate). Thus, although these cells had passed from metaphase into anaphase, they were terminally arrested and did not become viable G_1 cells as did wild-type cells, for which the mitotic percentage dropped from 36% (180 min) to 12% (270 min).

In both wild-type and *smc6-74* strains, cohesin was lost from all loci on undamaged chromosomes in cells not pretreated with HU (Fig. 5D), in keeping with the observation that *smc6-74* cells are wild type without extrinsic DNA damage (75). The magnitude of cohesin loss was greater in untreated *smc6-74* cells than in untreated wild-type cells, though we do not know the significance of this. This greater magnitude of loss required the inactivation of Mis4 at 36°C, since asynchronous cultures of *mis4-242* and *smc6-74 mis4-242* cells grown at 25°C (with Mis4 active) show similar levels of cohesin at all loci (Table 2). Further, while more cohesin remained at the centromeres in HU-treated *smc6-74* than in wild-type cells, these levels were <50% of untreated levels and were not sufficient to prevent kinetochore separation (Fig. 3A). We conclude that *smc6-74* cells are defective in the removal of cohesin from chromosome arms that have suffered DNA damage in the preceding interphase and that this retained cohesin prevents sister chromatid arm separation at anaphase.

Cohesin is recruited to a DSB in *smc6-74* cells. Cohesin is required for DSB repair, where it is proposed to facilitate recombination between sister chromatids by holding them in close proximity (66). siRNA studies of human cells have suggested that Smc5-Smc6 is required for cohesin recruitment to an I-SceI-induced DSB (57). This would be an ideal explanation for the sister-chromatid recombination defects caused by Smc5-Smc6 mutants or Smc5-Smc6 siRNAs. However, a failure of sister chromatids to interact is difficult to reconcile with the retention of cohesin shown here, the formation of recombination intermediates in *smc6* mutants of *S. pombe* (2), and the observation that *smc6* mutants in *S. cerevisiae* are not defective in recruiting cohesin to a DSB (65).

Given the differences between these studies of human cells and the yeasts, we next assessed whether Smc5-Smc6 is required for the recruitment of cohesin to a DSB in *S. pombe*. The site-specific HO endonuclease has been utilized in *S. pombe* to generate a DSB at an integrated *MAT***a** site (58). However, due to the absence of rapidly inducible promoters in *S. pombe* and due to the efficiency of DSB repair by HR, few cells contain a DSB at any one time. We isolated a ts allele of

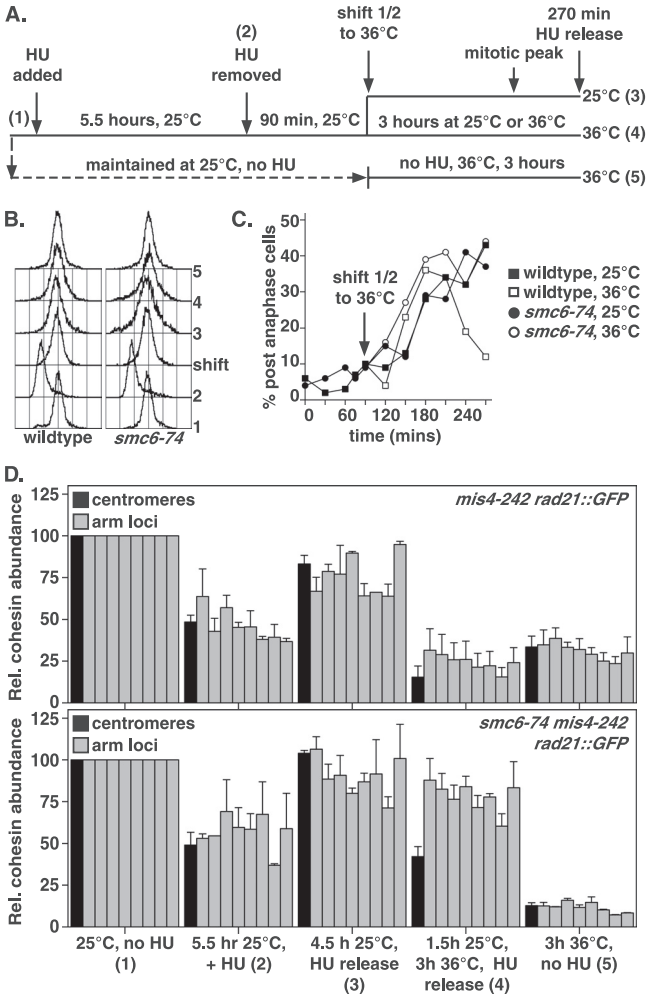

FIG. 5. Smc5-Smc6 is required for the removal of cohesin from damaged chromosomes. (A) Schematic of the time course used for preparing samples for ChIP of GFP-Rad21, using the ts *mis4-242* background. (B) FACS profiles of DNA contents of cultures used for cohesin ChIP. Numbers refer to the time points in panel A. (C) Mitotic progression of the cultures used for cohesin ChIP. Data are percentages of cells that become binucleate, cut, or incompletely resolved. The point where half the culture was shifted to 36°C (corresponding to "shift" in panel B) is indicated. The kinetics of entry into anaphase are the same for wild-type and *smc6-74* cells, but 75% of mitotic *smc6-74* cells fail to resolve their chromosomes and are cut or incompletely resolved, and hence the mitotic index does not decrease. (D) ChIP values (*n*-fold enrichment of cohesin over levels in an untagged control) normalized to those of asynchronous cultures grown at 25°C (time point 1). Loci are the *otr* of the centromeres (filled bars) and the loci on the chromosome arms (from left to right, 1L, 1R, 2L, 2R.1, 2R.2, 2R.3, 3L, and 3R) (shaded bars). Note the persistence of cohesin postanaphase in *smc6-74* cells following HU treatment and Mis4 inactivation (time point 4) compared to levels in the control without HU (time point 5). Data are means ± SE ($n = 3$). The *y* axis shows normalized *n*-fold enrichment of GFP-Rad21 over levels in an untagged control. Rel., relative. ChIP oligonucleotides and raw data are presented in Tables 1 and 2, respectively.

HO, which contains a point mutation (G401E) in the nuclease domain. Expression of this ts HO at 36°C from the *nmt1* promoter enables the stockpiling of (inactive) HO, which, upon a shift to 25°C for 2 h, results in ~20% cleavage at *MAT***a**, as determined by Southern blotting (Fig. 6B). Since ~80% of an asynchronous culture of *S. pombe* is in G_2 or mitosis with replicated sister chromatids, this corresponds to DSBs in ~40% of cells (Fig. 6A and B). Using Rad21-GFP ChIP, we observed 1.5- to 2-fold enrichment of cohesin on both sides of the DSB but not at a control locus (Fig. 6C; Table 3). Considering that only ~20% of chromatids are cleaved, this corresponds to 7.5- to 10-fold enrichment of cohesin at the DSBs over that already present without cleavage. This enrichment was essentially identical in wild-type and *smc6-74* cells, and thus we conclude that, as in *S. cerevisiae*, Smc5-Smc6 is not required for the recruitment of cohesin to chromosomal lesions in *S. pombe*.

Separase overexpression suppresses the aberrant retention of cohesin on postanaphase chromosome arms following DNA damage in *smc6-74* cells. Since Cut1 overexpression suppressed the aberrant mitoses of *smc6-74* cells upon recovery from an HU arrest, we assayed whether this also suppressed the retention of cohesin on chromosome arms. To this end, we repeated the ChIP experiments for which results are shown in Fig. 5 in cells overexpressing Cut1 (Fig. 7). Cut1 overexpression suppressed the block to segregation seen in Fig. 5C, with chromosomes now resolving as in wild-type cells (Fig. 7C). Not surprisingly therefore, Cut1 overexpression also suppressed the retention of cohesin following recovery from HU arrest (Fig. 7D, condition 4). Reproducibly, *smc6-74 mis4-242* cells overexpressing Cut1 also showed low levels of cohesin on chromosomes, even at 25°C. However, this must be an effect of the HU block and release protocol, since Cut1 had little effect on cells without HU treatment, and Cut1 overexpression restored growth to *smc6-74* cells chronically exposed to HU (Fig. 4).

Aberrant retention of cohesin on undamaged postanaphase chromosome arms in *top2-191 smc6-74* cells. We used the same ChIP protocol for cells containing the *mis4-242* allele that were grown at 25°C or shifted to 30°C for 4 h. Cohesin was also retained in postmitotic *smc6-74 top2-191 mis4-242* cells at 30°C, a condition lethal for *smc6-74 top2-191* cells and a significant impediment to the cohesin-loading capacity of *mis4-242* cells. For *top2-191 mis4-242* cells, >50% of cohesin was lost at 30°C. In contrast, *smc6-74 top2-191 mis4-242* cells retained cohesin to >90% of the levels seen at 25°C at all loci (Fig. 8A; Table 4), including the centromeres, where spindle forces may be sufficient to separate kinetochores (Fig. 3) with

TABLE 2. Enrichment of Rad21-GFP over levels in an untagged control for the normalizing data point for Fig. 5[a]

Locus (closest gene)	Fold enrichment of Rad21-GFP (mean ± SE) in a strain with the following genotype:	
	Wild type	*smc6-74*
Centromeres (*otr*)	90.3 ± 3.4	115.7 ± 14.0
1L (*pac2*)	49.6 ± 2.9	35.7 ± 1.9
1R (*fun14*)	34.5 ± 0.4	33.6 ± 1.8
2L (*act1*)	28.5 ± 0.6	29.1 ± 0.5
2R.1 (*zfs1*)	22.9 ± 2.6	25.6 ± 1.9
2R.2 (*pyr1*)	35.3 ± 1.8	29.9 ± 1.3
2R.3 (SPBP4H10.14)	39.4 ± 5.9	32.2 ± 3.6
3L (SPCC553.10)	41.7 ± 4.3	41.6 ± 2.6
3R (*ade6*)	34.3 ± 2.2	32.2 ± 6.3

[a] Growth at 25°C without HU.

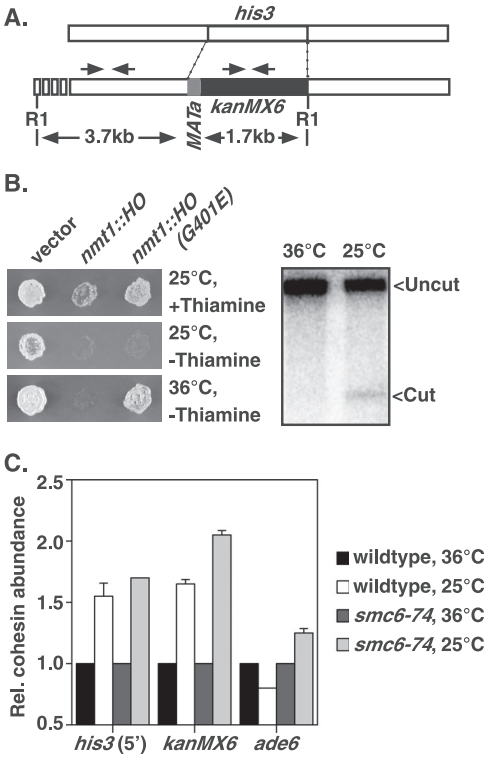

FIG. 6. Smc5-Smc6 is not required for cohesin recruitment to a DSB. (A) Schematic of the DSB assay system. A cassette containing the HO endonuclease recognition site (*MAT***a**::*kanMX6*) was used to replace the *his3* locus. R1 denotes EcoRI sites that flank the locus at the indicated distances. Arrows indicate the positions of ChIP primers used in the experiment for which results are shown in panel C. (B) Characterization of a ts HO allele expressed from the *nmt1* promoter. (Left) Plates show expression in a repair-defective *rhp51*Δ background. Expression of ts HO(G401E) is tolerated at 36°C, as well as at 25°C when the promoter is repressed (+ Thiamine), but is lethal at 25°C when the promoter is derepressed (− Thiamine). (Right) Southern blotting of EcoRI-digested genomic DNA from cells grown at 36°C (− Thiamine, promoter derepressed) or shifted to 25°C for 2 h and then probed with *kanMX6*. The HO-cut fragment represents 19% of the total signal measured in arbitrary phosphorimage units, corresponding to 38% of chromatids. (C) Cells were grown as for the Southern blot analysis for which results are shown in panel B and were processed for Rad21-GFP ChIP. The *ade6* locus is an undamaged site that serves as a negative control. Data are means ± SE ($n = 3$), normalized to 36°C cultures. ChIP oligonucleotides and raw data are shown in Tables 1 and 3, respectively. The *y* axis shows normalized *n*-fold enrichment of GFP-Rad21 over levels in an untagged control. Rel., relative.

cohesin present. As with the suppression of synthetic lethality, Cut1 overexpression also suppressed the retention of cohesin in *top2-191 smc6-74* cells at 30°C (Fig. 8B). *top2-191* cells showed the most significant loss of cohesin at 30°C, which may be the reason for the synthetic lethality seen with Cut1 overexpression in *top2-191* single mutants grown on plates at 30°C (Fig. 4B).

We conclude that Smc5-Smc6 is also required for the removal of cohesin from undamaged chromosomes when a noncatalytic Top2 mitotic function is attenuated. Therefore, Smc5-Smc6 and Top2 may cooperate in the structural integrity of mitotic chromosome arms necessary for the timely removal of cohesin.

DISCUSSION

Most DNA repair genes are not required for cell viability without extrinsic DNA damage. This likely reflects significant redundancy between the multiple repair pathways. Combining a DNA repair defect with a DNA damage checkpoint defect does lead to lethality, and since Smc5-Smc6 is implicated in both repair and checkpoint maintenance, we had previously proposed that this was the rationale for the essential nature of the Smc5-Smc6 complex (27, 75).

However, although Smc5-Smc6 is studied for its role in DNA repair, the terminal phenotype of Smc5-Smc6 mutants is mitotic failure (27, 75). Similarly, *S. cerevisiae smc5* mutants show chromosome loss and fragmentation (15). The data presented in this study show that the persistence of cohesin on damaged chromosome arms beyond anaphase is the major cause of mitotic failure in *smc6-74* cells. Importantly, this extends to chromosomes in *smc6-74 top2-191* double mutants, which, by several independent assays, are devoid of detectable DNA damage. Several other Smc5-Smc6 complex mutants show mitotic failure after DNA damage (3, 27, 41, 43, 45, 46) or when combined with *top2-191* mutants (Fig. 1), indicating that this defect reflects a requirement for a functional Smc5-Smc6 holocomplex. We see no requirement for Smc5-Smc6 in the recruitment of cohesin to a DSB, as has been proposed by RNA interference studies of human cells (57), and this is consistent with data from *S. cerevisiae* (65). Thus, the mitotic failure is in keeping with the late role for Smc5-Smc6 in HR (2, 43) and indicates that, as with cohesin and condensin, the Smc5-Smc6 complex executes an essential role in chromosome dynamics that is necessary for the cohesin regulators to remove cohesin complexes from chromosome arms prior to anaphase. We do not propose that Smc5-Smc6 is necessarily affecting cohesin removal directly but rather that Smc5-Smc6 is affecting chromosome structure in such a way that cohesin persists on chromosome arms beyond anaphase.

The chromosome segregation defects in *smc6-74 top2-191* mutants in the absence of DNA damage, catenations, or a condensation defect also support a chromosome structure effect on cohesin dynamics rather than the hypothesis that the defective removal of cohesin is a consequence of entering mitosis prior to the completion of DNA repair. Importantly, the synthetic lethality of *smc6-74 top2-191* mutants was rescued by expression of a Top2 mutant in which the catalytic tyrosine is mutated to phenylalanine (Y835F). Top2 is a dimer, but it is unlikely that the Y781F mutation suppresses *top2-191* within a dimeric Top2-191–Top2-Y835F molecule, since the catalytic

TABLE 3. Enrichment of Rad21-GFP over levels in an untagged control for the normalizing data point for Fig. 6[a]

Locus	Fold enrichment of Rad21-GFP (mean ± SE) in a strain with the following genotype:	
	Wild type	*smc6-74*
his3 (5′ to *MAT***a**)	8.6 ± 0.7	5.6 ± 1.2
kanMX6 (3′ to *MAT***a**)	6.4 ± 0.4	4.0 ± 0.9
ade6	23.4 ± 2.2	15.2 ± 2.2

[a] Time zero, 36°C.

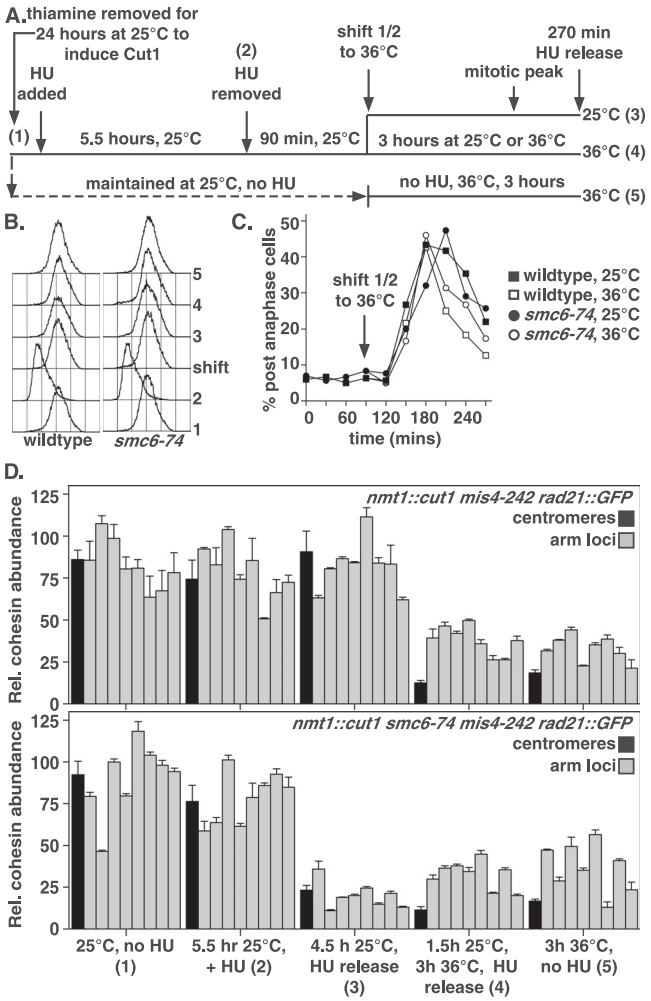

FIG. 7. Separase overexpression suppresses the cohesin retention defect of *smc6-74* cells. (A) The same protocol as that described for Fig. 5A was used to prepare cells, but cells were grown in the absence of thiamine for 24 h to induce Cut1 expression. (B) FACS profiles of DNA contents of cultures used for cohesin ChIP. Numbers refer to the time points in panel A. (C) Mitotic progression of the cultures used for cohesin ChIP. Data are percentages of cells that become binucleate, cut, or incompletely resolved. The point where half the culture was shifted to 36°C (corresponding to "shift" in panel B) is indicated. The delayed exit of *smc6-74* cells from mitosis, due to failed chromosome segregation (Fig. 5), was suppressed by Cut1 overexpression. (D) Anti-GFP ChIP values (*n*-fold enrichment over levels in an untagged control) normalized to those for asynchronous vector-only cultures grown at 25°C (time point 1). Primer sets and conditions are identical to those used for Fig. 5. Data are means ± SE ($n = 3$). Cohesin is no longer retained in *smc6-74* cells following recovery from HU arrest at 36°C (time point 4). Rel., relative.

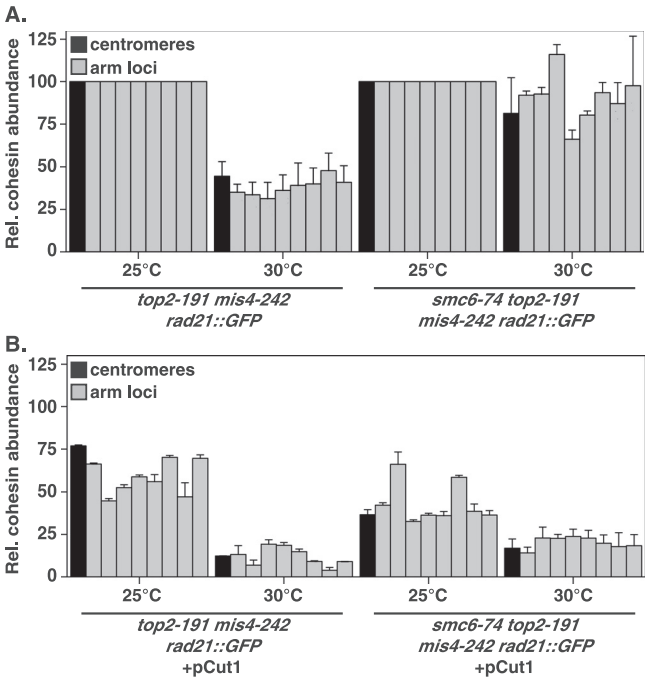

FIG. 8. Smc5-Smc6 is required for the removal of cohesin from undamaged chromosomes when Top2 function is compromised. (A) GFP-Rad21 ChIP (enrichment over levels in an untagged control) of samples grown at 25°C or shifted to 30°C. Data are normalized to levels for the 25°C samples. Note the persistence of cohesin at loci in *smc6-74 top2-191* cells at 30°C. Data are means ± SE ($n = 3$). The *y* axis shows normalized *n*-fold enrichment of GFP-Rad21 over levels in an untagged control. Rel., relative. ChIP oligonucleotides and raw data are presented in Tables 1 and 4, respectively. (B) GFP-Rad21 ChIP was performed as for panel A, but cells overexpressing Cut1 (Separase) were used, and data were normalized to those for vector-only controls. The cohesin retention observed in panel A for *top2-191 smc6-74* cells at 30°C is suppressed by Cut1 overexpression.

tyrosines of both subunits are required to interact with each strand of the G segment in the decatenation reaction (7, 78), although we cannot rule out a stabilizing effect on Top2-191. Therefore, it is likely that the defective removal of cohesin is a result of altered mitotic chromosome structure, where Top2 plays an important structural role in axial alignment independently of its function in decatenation (12, 14, 79). That is, a defect in Top2-mediated mitotic chromosome structure may be further exacerbated by the *smc6-74* mutation, and in combination the defect is too severe for chromosome segregation to occur.

The block to arm segregation that we describe here can be rescued by Separase overexpression. Importantly, the identification of Separase as a suppressor of the HU sensitivity of *smc6-74* mutants indicates that cohesin dysregulation is the

TABLE 4. Enrichment of Rad21-GFP over levels in an untagged control for the normalizing data point for Fig. 8[a]

Locus (closest gene)	Fold enrichment of Rad21-GFP (mean ±SE) in a strain with the following genotype:	
	top2-191	*smc6-74 top2-191*
Centromeres (*otr*)	45.8 ± 13.5	18.9 ± 4.2
1L (*pac2*)	52.6 ± 16.7	18.2 ± 4.8
1R (*fun14*)	37.1 ± 1.4	13.6 ± 2.0
2L (*act1*)	18.3 ± 0.6	6.6 ± 1.0
2R.1 (*zfs1*)	22.6 ± 0.2	11.1 ± 1.9
2R.2 (*pyr1*)	39.8 ± 1.3	19.8 ± 2.2
2R.3 (SPBP4H10.14)	28.3 ± 0.2	11.2 ± 1.3
3L (SPCC553.10)	26.4 ± 3.3	14.0 ± 1.3
3R (*ade6*)	21.4 ± 0.8	9.0 ± 0.3

[a] Growth at 25°C.

critical factor in the loss of *smc6-74* cell viability following replication stress. Since cohesin is as efficiently recruited to DSBs in *smc6-74* as in wild-type cells, it is also possible that the very dysregulation of cohesin removal at DSBs is the source of the late HR defect characteristic of Smc5-Smc6 mutants (2). However, the segregation defects following DNA damage may result from a global retention of cohesin throughout the chromosome arms (6, 73, 81), and not just at sites of DNA damage.

We propose that altered structure of mitotic chromosomes in Smc5-Smc6 mutants, either following DNA damage in interphase or combined with the noncatalytic Top2 defect, may impede the access of cohesin regulators necessary to promote cohesin removal. In view of the fact that the kinetochores do separate in these aberrant mitoses, and because cohesin removal is primarily defective on chromosome arms, it is most likely that it is the Separase-independent cohesin removal pathway that is dysfunctional in *smc6-74* cells. In human cells, this pathway is controlled in part by Scc3 phosphorylation (28) and the Wings Apart-like protein Wapl (24, 31), though the actual mechanism of cohesin removal is not yet known. Defects in this pathway significantly delay progression through anaphase, which eventually is enforced by Separase (24, 31, 48). Importantly, however, this eventual Separase rescue of chromosome segregation occurs in the presence of wild-type Smc5-Smc6, and so the block to sister chromatid separation described here in *smc6-74* cells appears to require elevated levels of Separase for resolution. That is, the overexpression of Separase enables it to cleave more cohesin complexes than in normal mitosis, and this bypasses a defect in cohesin removal resulting from a defective Separase-independent mechanism stemming from Smc5-Smc6 dysfunction.

Scc3 phosphorylation has not been characterized in *S. pombe*, and although the Wapl homolog Wpl1 destabilizes cohesin complexes in G_1 (8), its possible role in mitosis is not yet known. Thus, the mechanisms controlling Separase-independent cohesin removal will be the subject of further study. Once they are characterized, we will be in a position to ask if these processes are related to Smc5-Smc6 function.

Pds5 also regulates sister chromatid cohesion by maintaining cohesin on replicated chromosomes (26, 56). In findings related to our own, a noncatalytic role for Top2 in cohesin regulation has also been suggested by the high-copy suppression of lethal *pds5* mutations in *S. cerevisiae* (1) by the same catalytically inactive Top2 mutant that rescues the synthetic lethality of *top2-191 smc6-74* mutants of *S. pombe*.

Compared to those of other SMC complexes, the precise function of Smc5-Smc6 has proven difficult to elucidate. Our data highlight an essential mitotic function for Smc5-Smc6, required for the removal of chromosomal cohesin. Defects in this mitotic function are a major element of the lethality caused by Smc5-Smc6 dysfunction and are in keeping with the fact that defects in HR are not lethal. The mitotic defects manifest in hypomorphic mutants either after DNA damage or in combination with the *top2-191* mutation. The terminal phenotype of cells null for *smc6* or *nse1* is a similar mitotic failure (27, 75), though this is without extrinsic DNA damage and with wild-type *top2*. If null spores are germinated in the presence of DNA-damaging agents, they die in the first mitosis (27, 75). However, *smc6*Δ and *nse1*Δ cells successfully divide three to five times in the absence of DNA-damaging agents before this phenotype manifests, and this may give sufficient time for spontaneous damage to accumulate. It is therefore possible that Smc5-Smc6 provides an essential requirement to respond to intrinsic DNA damage and replication stress, where Smc5-Smc6 promotes repair by HR and coordinates this with cell cycle progression (checkpoint maintenance) and chromosome segregation (cohesin removal).

The cohesin complex was originally shown to be required for DNA repair based on the radiation sensitivity of the *S. pombe rad21* hypomorph, *rad21-45* (9, 10, 21). Similarly, the first *S. pombe smc6* hypomorph, *smc6-X*, was originally named *rad18-X* and came from the same collection of *rad* mutants as *rad21-45* (50). Thus, while required for DNA repair, Smc5-Smc6 should, like cohesin, be similarly considered an essential regulator of chromosome structure, defects in which manifest as DNA repair and checkpoint maintenance defects. Once we know its precise function, the Smc5-Smc6 complex can assume a more descriptive name.

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